UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

DIPLOMOVÁ PRÁCE

Úloha TAK1 v přežití neuronů

Vypracováno na
Institutu farmakologie
Oddělení molekulárni farmakologie
Ruprecht-Karls-University
Heidelberg

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2010 Jana Prokipová

CHARLES UNIVERSITY IN PRAGUE
FAKULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DIPLOMA THESIS

The role of TAK1 in neuronal survival

Performed at
Institute of Pharmacology
Department of Molecular Pharmacology
Ruprecht-Karls-University
Heidelberg

Supervisors:

Prof. Dr. Markus Schwaninger
Doc. MUDr. Radomír Hrdina, CSc.

2010 Jana Prokipová

I declare that this work is my original author work, which I had developed by myself. All literature and other sources, which I had used, all of them are given in the list of used literature and they are quoted in text regularly.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a jsou v práci řádně citovány.

.....
Jana Prokipová

ACKNOWLEDGEMENT

I would like to thank to my supervisor in Germany, Prof. Dr. Markus Schwaninger for accepting me and for his support with my diploma thesis.
I would like to express thanks to Melanie Neubert, who assisted me and guided me throughout my whole stay in Germany. I am thankful for her care, friendship, help during my work in the lab and for teaching me many methods. I really appreciate it.
Further, I would like to thank my supervisor in Czech Republic, Doc. MUDr. Radomír Hrdina, CSc., for his advice to this work and his patience.

Thank you all,

Jana

Ráda by jsem poděkovala mému školiteli v Německu, Prof. Dr. Markusovi Schwaningerovi za to, že mne přijal do jeho pracovní skupiny a za jeho podporu při psaní mé diplomové práce.
Dále bych ráda poděkovala Melanii Neubert, která mi asistovala a vedla mě počas celého mého pobytu v Německu. Jsem velmi vděčná za její péči, přátelství, pomoc s prací v laboratoři a za to, že mě naučila spoustu metod. Velmi si toho vážím.
V neposlední řadě bych ráda poděkovala mému školiteli v České republice, Doc. MUDr. Radomírovi Hrdinovi, za jeho rady k mé diplomové práci a za jeho trpělivost.

Děkuji vám všem,

Jana

ABSTRACT

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Performed at
Institute of Pharmacology
Department of Molecular Pharmacology
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Title of diploma thesis: The role of TAK1 in neuronal survival

Transforming growth factor-β activated kinase-1 (TAK1) is a serine/threonine kinase and it is part of the mitogen-activated protein kinase (MAPK) signaling. TAK1 is a key modulator of the transcription factors NF-κB and AP1. Recent studies have shown that TAK1 is essential for the survival of different cell types. Here, we focus on the biological role of TAK1 in neurons *in vivo*. We used DAB immunohistochemistry to evaluate the effect of TAK1 in neuronal survival. Therefore we compared the number of neurons in TAK1 knockout mice and $TAK1^{fl/fl}$ control mice. However, we did not find any significant difference in the number of neurons between both groups of mice.

ABSTRAKT

Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra farmakologie a toxikologie

Vypracováno na
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Název diplomové práce: Úloha TAK1 v přežití neurónů

TAK1 je serin/threoninová kináza, která je členem proteinové rodiny MAPK. TAK1 je klíčový modulátor transkripčních faktorů NF-κB a AP1. Nedávné studie prokázaly, že TAK1 je nezbytný pro přežití určitých typů buněk. V této práci jsme se zaměřili na biologickou roli proteinu TAK1 pro přežití neuronů. Porovnávali jsme počet neuronů u geneticky upravených myší, kterým chybí TAK1 s počtem neuronů u kontrolních myší. Avšak, nenašli jsme podstatný rozdíl počtu neuronů mezi oběmi skupinami myší.

ABBREVIATIONS

°C	degree Celsius
AP1	activator protein
IKK	IκB kinase complex
IL-1	interleukin-1
IL-1βR	interleukin-1 receptor
IκB	inhibitor of kappa B
JNK	c-Jun NH_2-terminal kinase
MAPK	mitogen-activated protein kinase
NEMO	nuclear-factor-kappa-beta essential modulator
NF-κB	nuclear-factor-kappa-beta
RHD	REL-homology domain
RIP1	receptor interaction protein 1
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAB1	transforming growth factor-β activated kinase-1 bindings protein 1
TAB2	transforming growth factor-β activated kinase-1 bindings protein 2
TAB3	transforming growth factor-β activated kinase-1 bindings protein 3
TAK1	transforming growth factor-β activated kinase-1
TGF-β	transforming growth factor-β
TLR	toll like receptor
TNF	tumor necrosis factor
TRADD	TNF receptor-associated death domain protein
TRAF2	TNF receptor associated factor 2
TRAF5	TNF receptor associated factor 5
TRAF6	TNF receptor associated factor 6
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
UV	ultraviolet

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1. INTRODUCTION

1.1.Stroke

Stroke is the third leading cause of death in the United States (Nestler et al.2009). It is a worldwide problem and causes vast medical, economical and personal costs.
Stroke, also known as cerebrovascular accident, occurs when blood flow to the brain is interrupted. It is either caused by obstruction or hemorrhage. The brain loses its energy supply, which results in oxygen and glucose deprivation. Since neurons are vulnerable to energy deprivation this can lead to a complete loss of function within ten seconds (Nestler et al.2009).

1.2.Neuronal cell death

Due to deprivation of essential nutrients neurons enter a stress state and the membrane potential breaks down. When neurons depolarize they transmit action potentials and within this reaction they release glutamate into the synaptic cleft. This initiates a destructive cycle of activation of neighboring cells and further glutamate release. Due to the excitotoxic effect of this self-enhancing glutamate release, ion gradients disrupt, calcium flows into the cell, the cells swell, mitochondrial membranes get damaged, cellular proteases and lipases get activated and free radicals accumulate. Finally, all these processes will result in the death of the neuron (Nestler et al.2009).
Depending on the strength of the harmful stimulus during ischemia, neurons will either die by a programmed cell death, called apoptosis or by cellular disruption, called necrosis. The infarct area after ischemia can be divided in two major zones, the infarct core, where blood supply is cut almost completely and cells receive a very harmful stimulus that will induce necrosis and the surrounding area, the so called penumbra where cells receive a milder stimulus and may enter apoptosis (Nestler et al.2009).

1.2.1. Necrosis

In response to cellular stress, such as ischemia, UV irradiation, free radicals, toxins, neurons activate intracellular stress pathways. When neurons receive excessive injury, these pathways are over-activated. The resulting necrotic neurons and their organelles swell, or burst. Necrosis is known for the leakage of cytoplasmatic and organelle contents into the extracellular space. This might be toxic to neighboring cells and can lead to inflammation. Necrosis is always a detrimental process (Nestler et al.2009).

1.2.2. Apoptosis

Apoptosis is a process of programmed cell death. It is already important during development of nervous system, because it causes pruning of excess neurons and corrects connections between nerve cells. On the other hand, apoptotic mechanisms of neuronal death contribute to neurodegenerative disorders (Nestler et al.2009).

Apoptotic neurons are subjected to a series of tightly regulated processes. Unlike necrosis, during apoptosis potentially dangerous intracellular material can safely be liquidated. Such material is packaged into vesicles, released and can be taken up by endocytosis and metabolized by surrounding cells. Apoptosis is an active process and results from the execution of a well-regulated genetic program.

Deleterious effect of apoptosis can be induced by activation of stress-activated protein kinases like c-Jun NH_2-terminal kinase (JNK) or p38/MAPK (Nestler et al.2009). Also Nuclear-factor-kappa-beta (NF-κB) has been shown to be a primary regulator of programmed cell death. NF-κB is well known for its anti-apoptotic function (Barkett and Gilmore 1999; Dutta et al. 2006). However, there is also evidence for its pro-apoptotic action (Qin et al. 1999; Pizzi et al. 2002). Transforming growth factor-β activated kinase-1 (TAK1) is an upstream signal of JNK, p38/MAPK and NF- κB and might therefore play a role in regulation of apoptosis.

1.3.Transforming growth factor-β activated kinase-1

The serine/threonine kinase Transforming growth factor-β activated kinase-1 (TAK1) is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family and is involved in many signaling pathways in cells (Yamaguchi et al. 1995).

Activity of TAK1 requires its binding proteins TAB1, TAB2 and TAB3 (Shibuya et al. 1996; Takaesu et al. 2000; Cheung et al. 2003; Ishitani et al. 2003).

TAB1 and TAB2 proteins were identified in a yeast two-hybrid screen. TAB1 can activate TAK1 directly *in vitro*. It has been shown, that TAB1 enhances TAK1 kinase activity when co-expressed. TAB1 binds specifically to TAK1 (Shibuya et al. 1996) and induces autophosphorylation of TAK1 (Kishimoto et al. 2000). The role of TAB1 was investigated in different signaling pathways upstream of TAK1. TAB1 is dispensable for tumor necrosis factor- (TNF) and interleukin- (IL) induced activation of TAK1. Association of TAB1 and TAK1 is important for osmotic stress-induced activation of TAK1 (Inagaki et al. 2008).

TAB2 links TAK1 to TNF receptor associated factor 6 (TRAF6) and thereby mediates TAK1 activation in response to IL-1 (Takaesu et al. 2000). TAB3 mediates interaction of TAK1 with TRAF2 and TRAF6 in an IL-1 and TNF-dependent manner (Ishitani et al. 2003). Activation of the TAK1/TAB2 (or TAB3) complex requires K63-linked polyubiquitination in the presence of ubiquitin-conjugating enzyme E1, E2 (Ubc13 and Uev1A) and E3 (TRAF6) (Wang et al. 2001)

Taken together, TAB2 and its close homologue TAB3, unlike TAB1, does not activate TAK1 directly *in vitro.* Nevertheless, TAB2 and TAB3 play a critical role in activation of the IL-1- and TNF-induced NF-κB pathway, JNK and p38/MAPK (Ishitani et al. 2003).

1.4.TAK1-signalling pathways

TAK1 was originally identified as a kinase involved in transforming growth factor beta (TGF-β) signaling pathway and a regulator of bone morphogenetic protein signals (Yamaguchi et al. 1995). Stimulation of cells with pro-inflammatory cytokines such IL-1β and TNF initiates a cascade of signaling pathways, including activation of NF-κB and MAPKs such as c-Jun NH_2-terminal kinase (JNK) and p38/MAPK. It induces the expression of genes in the nucleus that regulate inflammation (Dinarello 1996). As shown in Figure 1, TAK1 was found to be a crucial regulator of these pathways (Ninomiya-Tsuji et al. 1999; Takaesu et al. 2000; Wang et al. 2001).

Under physiological conditions, TAK1 is associated with transforming growth factor-β (TGF-β) type I receptor (TβRI) through a complex with TAB2 and the ubiquitin ligase TRAF6. Upon TGF-β stimulation, TβRI and TβRII form a heterotetrameric comlex, which leads to autopolyubiquitination of TRAF6 and subsequent polyubiquitination of TAK1. TAK1 in turn dissociates from TβRI. After release of TAK1 from receptor complex, TAK1 interacts with TAB1, which induces autophosphorylation of TAK1 (Kim et al. 2009). Then, TAK1 triggers downstream signaling cascades, including MAPK kinase (MKK) 4/7- JNK cascade, MKK 3/6-p38/MAPK cascade and activation of NF-κB (Kishimoto et al. 2000). JNK and p38/MAPK control activation of the transcription factor activator protein (AP1) (Chen and Goeddel 2002).

Stimulation of IL-1β receptor1β (IL-1βR) and toll like receptor (TLR) leads to activation of TRAF6. TRAF6 causes K-63 polyubiquitination (Deng et al. 2000), which leads to activation of TAK1 kinase complex. TAK1 activates IκB kinase (IKK) complex (Wang et al. 2001) that in turn phosphorylates the IκB proteins and thereby targets this inhibitor of NF-κB activity for proteosomal degradation. Now, free NF-κB can enter the nucleus and modulate target gene expression (Scheidereit 2006).

In the TNF pathway, binding of TNF to its receptor leads to the recruitment of signal proteins such as TRADD, TRAF2, TRAF5, and receptor interaction protein 1 (RIP1). Polyubiquitinated RIP1 is essential for TNF signaling and recruits IKK through the interaction between polyubiquitin chains and NEMO (Ea et al. 2006). Polyubiquitination of RIP1 recruits the TAK1 complex through the interaction between polyubiquitin chains and TAB2 or TAB3 (Kanayama et al. 2004). It allows TAK1 to phosphorylate IKK and thus induce activation of NF-κB (Wang et al. 2001).

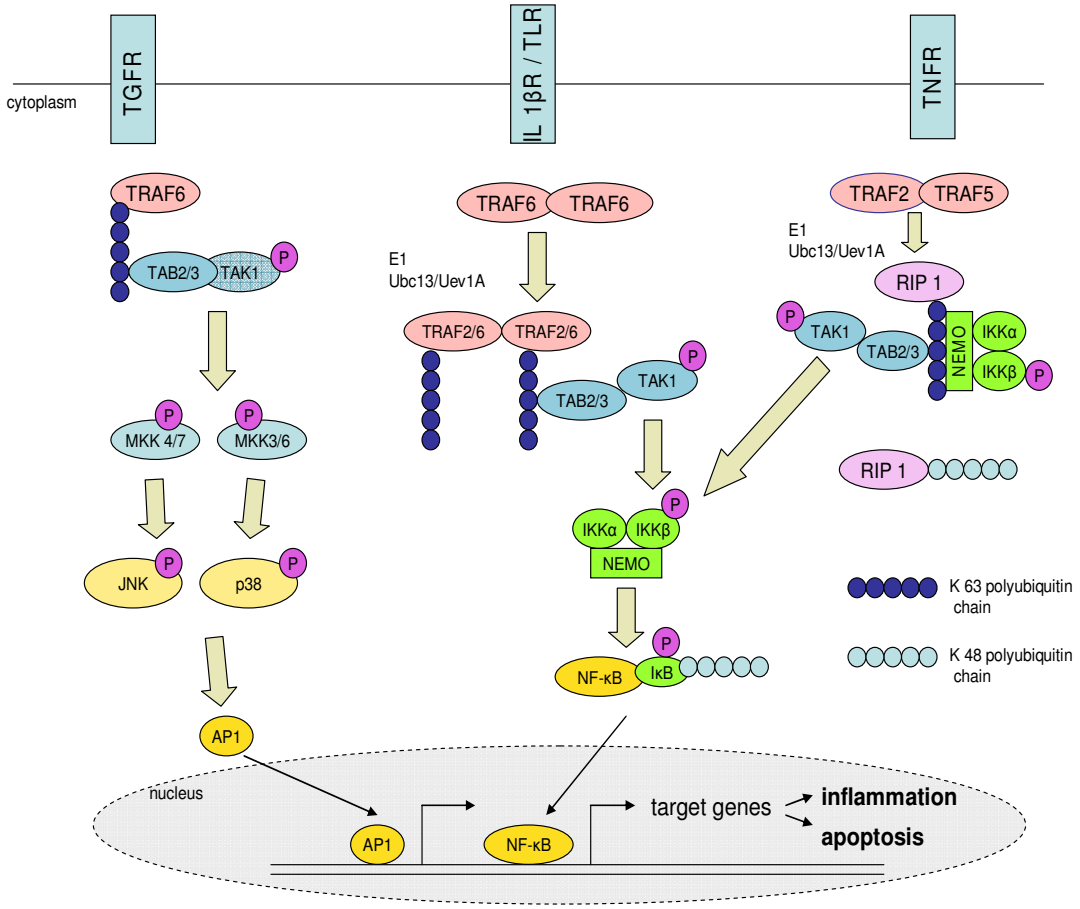


Figure 1: A model of TAK1 activation by stimulation of TGF receptor, IL-β receptor/Toll like receptor and TNF receptor

Exposure of cells to growth factors, pro-inflammatory cytokines leads to activation of specific ubiquitin ligases, which promote K-63 polyubiquitination. In case of TGFR, K-63 polyubiquitination attracts TAB2/3, which promotes activation of TAK1. TAK1 can phosphorylate MKK in the JNK, p38/MAPK pathway. JNK and p38/MAPK control the transcription factor AP-1.

Stimulation of IL-1βR/TLR leads to activation of TAK1. TAK1 then phosphorylates IKK complex that in turn phosphorylates IκB and targets this inhibitor for proteosomal degradation. Free NF-κB can enter the nucleus to modulate target genes.

Stimulation of TNFR leads to activation of TRAF2, TRAF5, which then promote K-63 polyubiquitination of RIP1. Polyubiquitination of RIP1 recruits TAK1 complex through the interaction between polyubiquitin chains and TAB2 or TAB3. The polyubiquitin chains also recruit IKK by binding to NEMO. This allows TAK1 to phosphorylate IKK and activate NF-κB.

1.5. The NF-κB signaling pathways

NF-κB is a key transcription factor that regulates biological processes including immunity, inflammation and apoptosis (Skaug et al. 2009). The NF-κB family of transcription factors consists of p50, p52, p65 (REL-A), c-REL, REL- B (for review, see Hayden and Ghosh 2008). These subunits form homo- and heterodimers in various combinations.

In the canonical pathway, NF-κB is activated by TNF, IL-1β, and TLR ligands that are recognized by specific membrane receptors. Stimulation of these receptors leads to activation of the TAK1 complex through TRAF proteins. TAK1 then activates IκB kinase complex (IKK), consisting of the two enzymatic subunits IKK1 (IKK-α) and IKK2 (IKK-β), and the regulatory subunit NF-κB essential modulator (NEMO) (Skaug et al. 2009). IκB (inhibitor of kappa B) proteins are bound to NF-κB dimers and retain the dimers in the cytosol. All IκB proteins are characterized by presence of ankyrin repeat domains that bind to NF-κB dimers. The N-terminal REL-homology domain (RHD), which is contained in all of the NF-κB family members, is responsible for homo- and heterodimerization, DNA binding, nuclear translocation, and interaction with IκB proteins (Skaug et al. 2009). Upon activation IKK phosphorylates IκB-α at serines 32 and 36, induces polyubiquitination of lysines 21 and 22 and finally degradation by the 26S proteasome (Chen 2005). After degradation of IκB, free NF-κB translocates into the nucleus and triggers gene transcription (for review, see Ridder and Schwaninger 2009).

NF-κB induces gene transcription of Bcl-2 family members with opposite effects, i.e. either pro-apoptotic (Bim, Noxa) (Inta et al. 2006) or anti-apoptotic (Bcl-2 and Bcl-X_L) (Tamatani et al. 1999). It has been shown, that NF-κB is activated in cerebral ischemia (Mattson and Camandola 2001; Inta et al. 2006). Pro-apoptotic Bcl-2 family members, Bim and Noxa, are up-regulated in cerebral ischemia. Interestingly, conditional deletion of RelA in neuronal cells or pharmacological inhibition of IKK blocked Bim and Noxa gene expression (Inta et al. 2006). On the other hand, protective effects of NF-κB may be mediated by c-Rel (Pizzi et al. 2002) Thus gene transcription regulates the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family members.

1.6. The role of TAK1 and its binding proteins in cell survival

TAK1-deficiency results in developmental abnormalities in the neuronal tube and embryonic death at day E9.5. This strongly suggests a critical role of TAK1 in early embryonic development (Shim et al. 2005). However, the phenotype of TAK1-deficient embryos is different from that of TAB1 and TAB2-deficient embryos. TAB1 mutant embryos die at a late stage of gestation due to failures in cardiovascular and pulmonary morphogenesis (Komatsu et al. 2002) and TAB2 mutant embryos die around embryonic day 12.5 due to liver apoptosis (Sanjo et al. 2003).

It has been shown that TAK1 is critical for the survival of both, hematopoietic cells and hepatocytes. Deletion of TAK1 results in bone marrow and liver failure due to apoptotic death of hematopoietic cells and hepatocytes. Furthermore, deletion of TAK1 results in inactivation of NF-κB and JNK signaling (Tang et al. 2008).

It has been reported that following activation by TNF and IL-1, mouse embryonic fibroblasts from TAK1-null mice exhibit decreased NF-κB and JNK activation. These cells are highly sensitive to TNF-induced apoptosis (Shim et al. 2005), whereas activation of NF-κB following TNF stimulation is required for protection of cells from TNF-induced cell death (Beg and Baltimore 1996; Van Antwerp et al. 1996). The conditional knockout system was used to delete RelA/p65 in the liver. In these mice hepatocyte apoptosis is induced upon stimulation with TNF (Geisler et al. 2007).

TAK1 is known to be critical for keratinocyte survival, since epidermal-specific deletion of TAK1 results in massive keratinocyte death due to sensitization to TNF-induced apoptosis. These cells fail to activate NF-κB or JNK upon TNF treatment. This suggests, that NF-κB and JNK play an important role in survival signaling upon TNF-induced apoptosis (Omori et al. 2006).

Taken together these studies confirm that NF-κB and JNK play a role in cell survival both after treatment with TNF and in the absence of any cytokine treatment. It has previously been demonstrated that NF-κB and JNK induce expression of both pro-apoptotic and pro-survival genes (Middleton et al. 2000; Pizzi et al. 2002; Liu and Lin 2005). There is evidence that both JNK and NF-κB are present in neurons (Wang et al. 2004; Zhang et al. 2005). To investigate whether TAK1 plays an important role in neuronal survival, we generated transgenic mice that were deficient for TAK1 in neurons.

1.7. Cre-lox system

Transgenic technology is an important tool for investigating individual gene function. Cre-lox recombination is a special type of site-specific recombination. This system was discovered in P1 bacteriophage. Cre-lox system can delete undesired DNA sequences.

It requires two components, first the Cre recombinase, an enzyme that catalyzes recombination between two loxP sites and additionally the loxP recognition sites (locus of X-over P1), consisting of 34 base pairs (8-base-pair core sequence flanked by 13-base-pair inverted repeats) (Sauer and Henderson 1988).

Cre recombinase is expressed under a tissue specific promoter. Mice carrying Cre recombinase are crossed with mice carrying loxP-flanked gene. The gene for Cre recombinase and loxP sites must be introduced to the mouse genome by transgenic technology (Nagy 2000) .

Cre recombination can induce a deletion, inversion, or chromosomal translocation depending on the orientation and location of the loxP sites. To induce deletion of target genes, loxP sites are required to be oriented in the same direction on a chromosomal segment (Nagy 2000) (Figure 2).

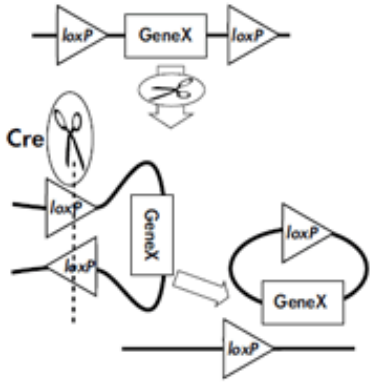


Figure 2: Orientation of loxP sites for deletion of target genes

Deletion of target genes occurs when loxP sites are oriented in the same direction on a chromosomal segment (www.jax.org).

2. AIM OF WORK

In this study we focus on the biological role of TAK1 in neurons *in vivo*.

In order to study the function of TAK1 in neurons, a transgenic mouse was generated that was deficient for TAK1 in neurons. Since global TAK1 deletion is embryonically letal, a conditional knockout strategy was chosen. Therefore $TAK1^{fl/fl}$ mice (Sato et al. 2005) were crossed with mice carrying the Cre recombinase under the promoter of CamKinase-II alpha (CamKII-a-Cre) (Casanova et al. 2001). CamKinase-II alpha promoter is expressed postnatally (Burgin et al. 1990). This strategy resulted in the generation of mice in which the floxed Exon II of the TAK1 gene was specifically deleted in neurons after birth.

We evaluated the effect of TAK1 on neuronal survival by comparison of the number of neurons in TAK1 knockout mice and $TAK1^{fl/fl}$ control mice.

To determine whether TAK1 deletion has an impact on neuronal survival, we investigated neurons in two different areas of the brain, the cerebral cortex and the hippocampus.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Animals

We used sixteen to twenty-seven-week-old age- and sex-matched male and female mice (Table 1). $TAK1^{fl/fl}$ mice were crossed with CamKII-alpha-Cre (CKC-cre) mice (Casanova et al. 2001). Half of the animals used for this study were positive for the CKC-cre allele ($TAK1^{nko}$) and CKC-cre negative littermates ($TAK1^{fl/fl}$) were taken as controls. All lines were backcrossed on a C57Bl/6 background.

Table 1: Mice of the experiment

	MALE	**FEMALE**
TAK1 neuronal knockout mice ($TAK1^{nko}$)	10	7
TAK1 floxed-control mice ($TAK1^{fl/fl}$)	12	5

3.1.2. Material

- Conical test tube PP (NP Nerbe Plus, Winsen/Luhe, GE)
- Cover slips (Carl Roth, Karlsruhe, GE)
- Fat pen (Dako Deutschland, Hamburg, GE)
- Microtube 1,5 ml (Sarstedt, Nümbrecht, GE)
- Pasteur pipette (Neolab, Heidelberg, GE)
- Pipette (Gilner Geiner Bio-One, Frickenhausen, GE)
- Pipette tips (Gilner Geiner Bio-One, Frickenhausen, GE)
- Polysine slides (Menzel Gläser, Braunschweig, GE)
- Serological pipette 5ml, 10 ml, 25 ml (Sarstedt, Nümbrecht, GE)
- Staining dish (Neolab, Heidelberg, GE)
- Tissue freezing medium (Jung, Nussloch, GE)

3.1.3. Instruments

- Leica cryotome CM 3050S (Leica, Mannheim, GE)
- Microscope leica DM LS2 (Leica, Mannheim, GE)

3.1.4. Chemicals

- Albumin fraktion V. (Carl Roth, Karlsruhe, GE)
- Distilled water (IPMB, GE)
- Glycerol anhydrous (AppliChem GmbH, Darmstadt, GE)
- H_2O_2 30% (CARL ROTH, Karlsruhe, GE)
- HCl fuming 37% (Merck KGaA, Darmstadt, GE)
- Horse serum (Invitrogen, Karlsruhe, GE)
- KCl (AppliChem GmbH, Darmstadt, GE)
- KH_2PO_4 (Grüssing GmbH, Filsum, GE)
- Methanol 100% (J.T.Baker, Deventer, NL)
- Mowiol 488 (Carl Roth, Karlsruhe, GE)
- Na_2HPO_4 x 2 H_2O (AppliChem GmbH, Darmstadt, GE)
- NaCl (VWR International GmbH, Darmstadt, GE)
- Paraformaldehyde (Sigma-Aldrich, Seelze, GE)
- Sucrose (Applichem, Darmstadt,GE)
- Triton X-100 (Merck, Darmstadt, GE)

3.1.5. Stock solution

PBS 10x

- 80.0 g NaCl
- 2.0 g KCl
- 18 g Na_2HPO_4 x $2H_2O$
- 2.4 g KH_2PO_4
- Add up 1000 ml with dH_2O
- Adjust pH to 7.4

1x PBS

- 100.0 ml PBS 10x
- Add up 1000 ml with dH_2O

3.1.6. Antibodies

- Anti-mouse biotinylated IgG (H+L), made in horse (Vector Laboratories, CA 94010, Lot: NO 923, Burlingame, CA)
- MsX NeuN MAB377 (Chemicon, CA 92 590, Lot: LV 1427917 , Temecula, USA)

3.1.7. Kits

- ABC Kit-Vectastain, Peroxidase standard (Vector Laboratories, Burlingame, CA)
- DAB Kit-Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA)

3.1.8. Imagine software

- Adobe Photoshop CS2
- Graph Pad Prism
- ImageJ
- Microsoft Office

3.2.Methods

3.2.1. Protein Analysis

3.2.1.1. SDS-PAGE analysis of proteins

To separate proteins of cell lysates, we performed Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). First, brains were isolated from the mouse. Then, cortex and cerebellum were isolated and immediately frozen on dry ice. Tissues were pestled on dry ice. SDS-sample buffer was added and samples were incubated for five min at 95°C, cooled down on ice and equally loaded onto a SDS-polyacrylamide gel with an acrylamide concentration of 8%. Gel electrophoresis was done at 100V.

3.2.1.2. Western Blot analysis

In order to detect a specific protein in a cell lysate, after separation of proteins by charge and size using SDS-PAGE, we blotted proteins onto a nitro-cellulose membrane. The membrane was equilibrated in blotting buffer and subsequently proteins were blotted onto the membrane at 300 mA for two hours in a wet-blot chamber.
Membrane was shortly washed in TBS-T and blocked in 5 % milk in TBS-T. Then membrane was washed three times for five minutes in TBS-T, following incubation in the first antibody (either anti-TAK1 or anti-tubulin) over night at 4°C. The next day membranes were washed three times for five minutes in TBS-T and then incubated for one hour at room temperature with the secondary anti-rabbit antibody (1:5000) in 5 % milk. Membrane was washed three times for five minutes in TBS-T. For detection, the membranes were exposed to enhanced chemiluminescence solution for one minute and then exposed to a film in the dark room.

3.2.2. Tissue preparation

- ANESTHESIA

Mice were anesthetized by injecting intraperitoneally 20 µl/g bodyweight of avertin. To confirm whether mice were anesthetized paw-reflex was tested.

- TRANSCARDIAL PERFUSION

After cutting off the muscle and bone to uncover the heart a small incision was made in the right ventricle of the heart and the mouse was perfused through left ventricle with 5 ml Ringer solution until the liver became white. Initially we started the experiment by subsequent perfusion of mice with 4% paraformaldehyde (PFA). After evaluation of cryosections this step was eliminated in further experiments due to morphological changes.

- DISSECTION OF THE BRAIN

Starting the experiment we dissected the brains and incubated them in PBS for 1h followed by incubation in 10% sucrose in PBS for 1h and 20% sucrose for 1h and finally in 30 % sucrose overnight. After evaluation of cryosections this step was modified such that brains were immediately frozen on dry-ice after dissection and frozen at -20 °C (for detailed overview on individual treatment see table below).

- CUTTING THE BRAINS

The brains were cut with a cryotome at -22°C to -25°C. Sections had a thickness of 20 µm and were collected on poly-lysine-coated-slides and subsequently stored at -20°C.

Table 2.: Individual treatment of the brains after dissection.

Mouse-line numbers refer to the mouse line in the mouse keeping unit: 0987: TAK1; 0918: TAK1-flox; 1193: TAK1-ZNSKO.

MOUSE-LINE	NAME	4% PFA IN PBS	SUCROSE	DRY ICE
918	37	X	X	
918	62	X	X	
918	102		X	
918	103		X	
987	446			X
987	447			X
1193	140			X
1193	143			X
1193	150			X
1193	152			X
1193	173			X
1193	174			X
1193	175			X
1193	183	X	X	
1193	184	X	X	
1193	192		X	
1193	193		X	
1193	194		X	
1193	197		X	
1193	198		X	
1193	199			X
1193	200			X
1193	201		X	
1193	202		X	
1193	203		X	
1193	204			X
1193	205			X
1193	206			X
1193	209			X
1193	210			X
1193	211			X
1193	212			X
1193	213			X
1193	219			X

3.2.3. Immunohistochemistry staining

3.2.3.1. Staining for NeuN with DAB

Slides were dried at room temperature for fifteen minutes and sections were surrounded with a fat- pen. Then slides were delipidate with 100% CH_3OH for ten minutes at -20°C. After drying at room temperature for about twenty minutes slides were rinsed three times for ten minutes in PBS. To block endogenous peroxidase activity slides were rinsed with 2% H_2O_2/10% CH_3OH/PBS for twenty minutes at room temperature. Afterwards sections were washed three times for ten minutes in PBS. Sections were permeabilized in 0.4 % Triton X-100/PBS for one hour and then blocked with 1.5 % horse serum/1% BSA/0.1% Triton X-100/PBS for one hour at room temperature. Then sections were incubated with anti-NeuN monoclonal antibody (1:1000 in 0.1% Triton X-100/1% BSA/PBS) at 4°C overnight. After that, sections were washed three times for ten minutes in PBS and incubated with secondary anti-mouse biotinylated IgG (1:50) in 2% horse serum/PBS. Then peroxidase-labeled avidin-biotin complex was incubated for thirty minutes. The ABC complex was prepared by mixing 2.5 ml PBS and 1 drop solution A and 1 drop solution B followed thirty minutes of incubation. Afterwards slides were washed three times for ten minutes in PBS. Sections were rinsed with DAB solution, which consist of 2.5ml distilled water with 1 drop buffer, 2 drops DAB, 1 drop hydrogen peroxide. DAB solution was applied until sections became dark. To stop DAB reaction, slides were washed with PBS for ten minutes and mounted with coverslips using Mowiol. Mowiol mounting medium consists of 2.4 g Mowiol 4-88/6.0g glycerol/6.0ml distilled water/12 ml 0.2 M Tris-HCl (pH 8.5).

3.2.4. Evaluation of cell number

Stained sections were photographed using a microscope at 5x or 10x magnification. Images were edited using the software Adobe Photoshop CS2. Brain structures were identified by overlapping the individual sections with a contour of a model section from a brain-atlas library (www.brain-map.org). This method assured that identical sections and brain-areas were evaluated. Cells were counted manually using the software ImageJ

and the cell counter Plug-in. Results were analyzed and statistically evaluated using Microsoft Excel and Graph Pad Prism.

4. RESULTS

4.1.Protein Analysis

In order to study the impact of TAK1 on neuronal survival *in vivo* we used a mouse line with a neuron specific deletion of the TAK1 gene ($TAK1^{nko}$) and control mice ($TAK1^{fl/fl}$). First we determined protein level of TAK1 in the cortex and cerebellum of both lines by Western Blot analysis and found a strong reduction of TAK1 protein level in the cortex of $TAK1^{nko}$ mice (Fig. 3) compared to $TAK1^{fl/fl}$ mice. The CaMKIIalpha promoter is not active in the cerebellum. We found no difference in the protein level of TAK1 in lysates of the cerebellum.

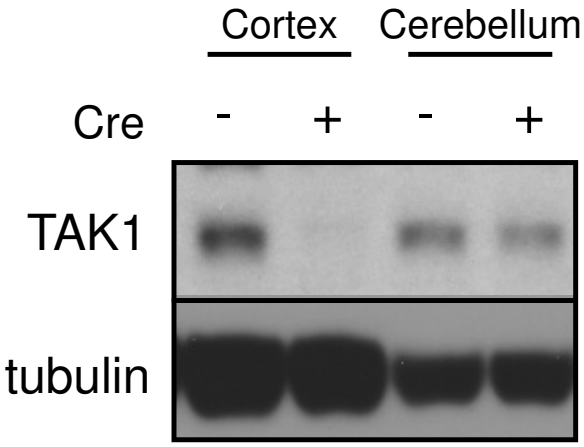


Figure 3: TAK1 protein is absent in the cortex, but not in the cerebellum of $TAK1^{nko}$ mice

Western Blot of protein lysates from mouse cortex or cerebellum. The protein levels of TAK1 in mice that express the Cre recombinase (+) under the CaMKII-alpha promoter ($TAK1^{nko}$) were compared to those not expressing Cre (-) ($TAK1^{fl/fl}$). Same loading of the samples was controlled by detection of tubulin protein levels on the same membrane.

4.2. Cerebral cortex

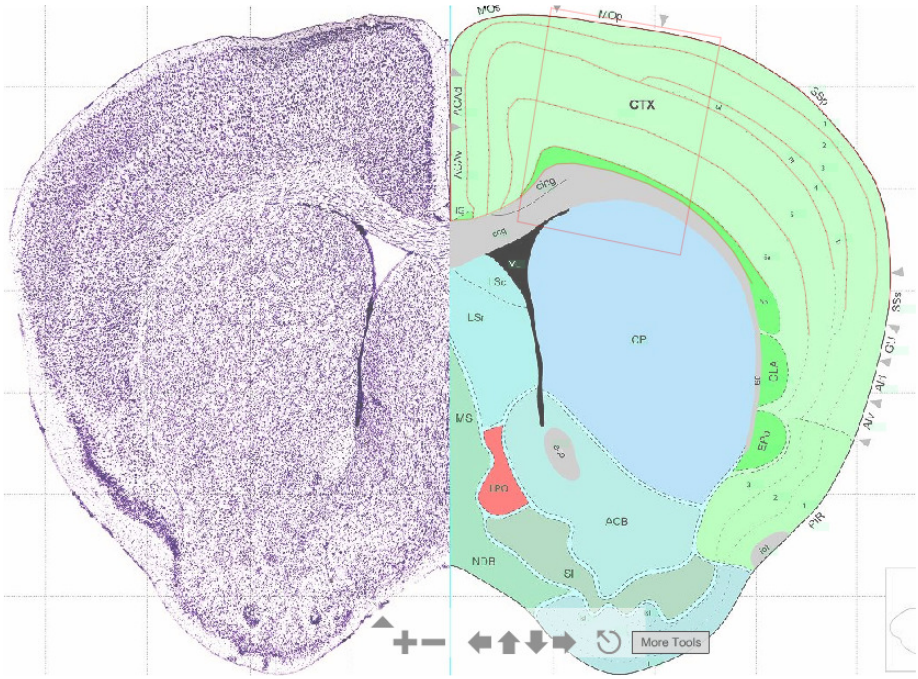
In order to analyze the impact of TAK1 on neuronal survival, we sought to quantify the amount of neurons in brains of $TAK1^{nko}$ and control mice. Since neurons specifically express the nuclear protein NeuN (Mullen et al. 1992) we used immunohistochemistry of NeuN to label neurons. A reference section from brain-atlas was taken and compared to cryosections of test mice (Fig.4, 5).

Quantification of neurons in the cortex at the level of section number 47 (number according to Paxinos Brain Atlas) revealed no difference in the numbers of neurons of $TAK1^{nko}$ and control mice (Fig. 6). All layers of the cortical sections were analyzed individually and no significant difference in neuronal numbers was observed. However, we found a trend that Cre+ mice seem to have lower number of neurons in all layers.

Additionally, we compared the total number of neurons in a determined area of the cortex and observed huge variation in the number of neurons in between the groups. Although Cre+ mice have lower number of neurons, no significant difference is observed between both groups (Fig. 7).

To evaluate whether the number of neurons in the individual layers between both, Cre- and Cre+ mice are different, we tested the groups in 2-way ANOVA (Figure 8). No significant difference was found between the individual layers and groups.

A.



B.

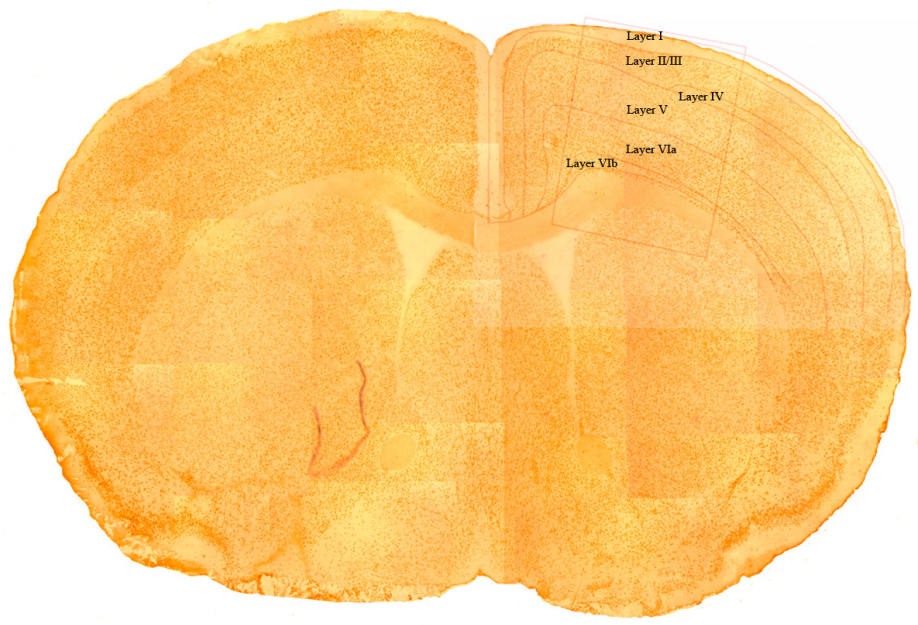


Figure 4. Used section for counting of neurons in cortex

(A) Model section from brain-atlas with contour of layers. (B) Stained section with anti-NeuN antibody (1:1000) and DAB solution. The cortex is overlapped with contour of a model section – number 47 according to Paxinos Brain Atlas). The neurons were counted in area of rectangle.

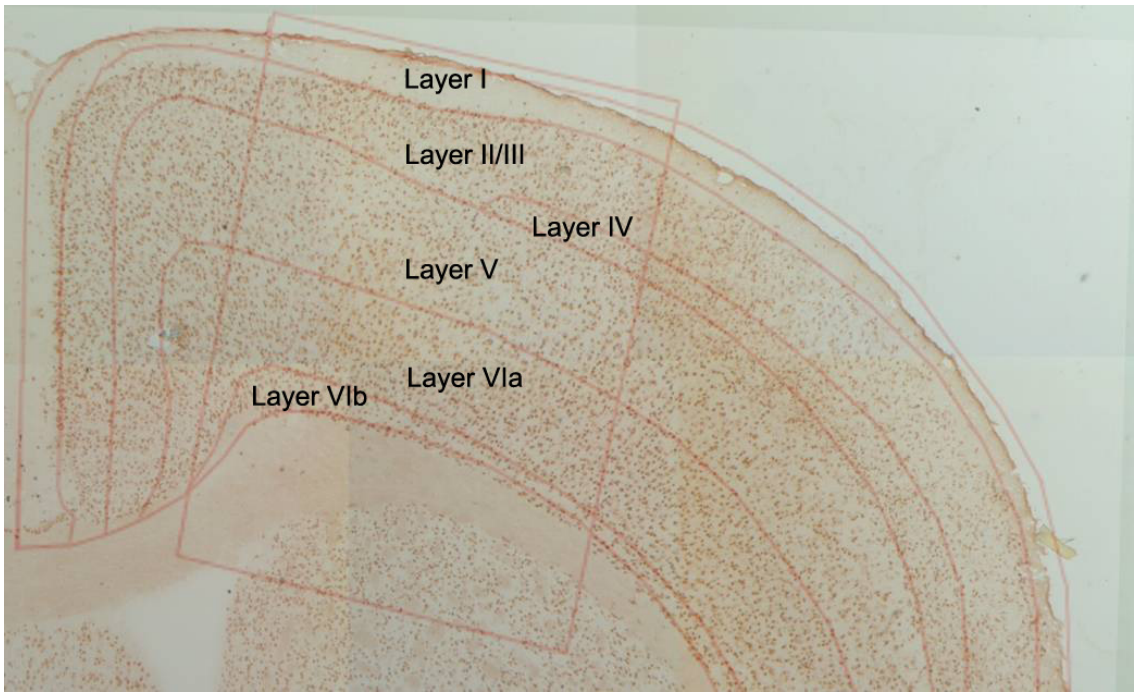


Figure 5. Example of stained section with antibody anti-NeuN (1:1000) by 5x magnification

DAB immunohistochemistry was used to visualize the neurons in cortex. The neurons were stained by using the anti-NeuN antibody (1:1000). Region of cortex is divided in Layer I, Layer II/III, Layer IV, Layer V, Layer VIa and Layer VIb. The neurons were counted in area of rectangle.

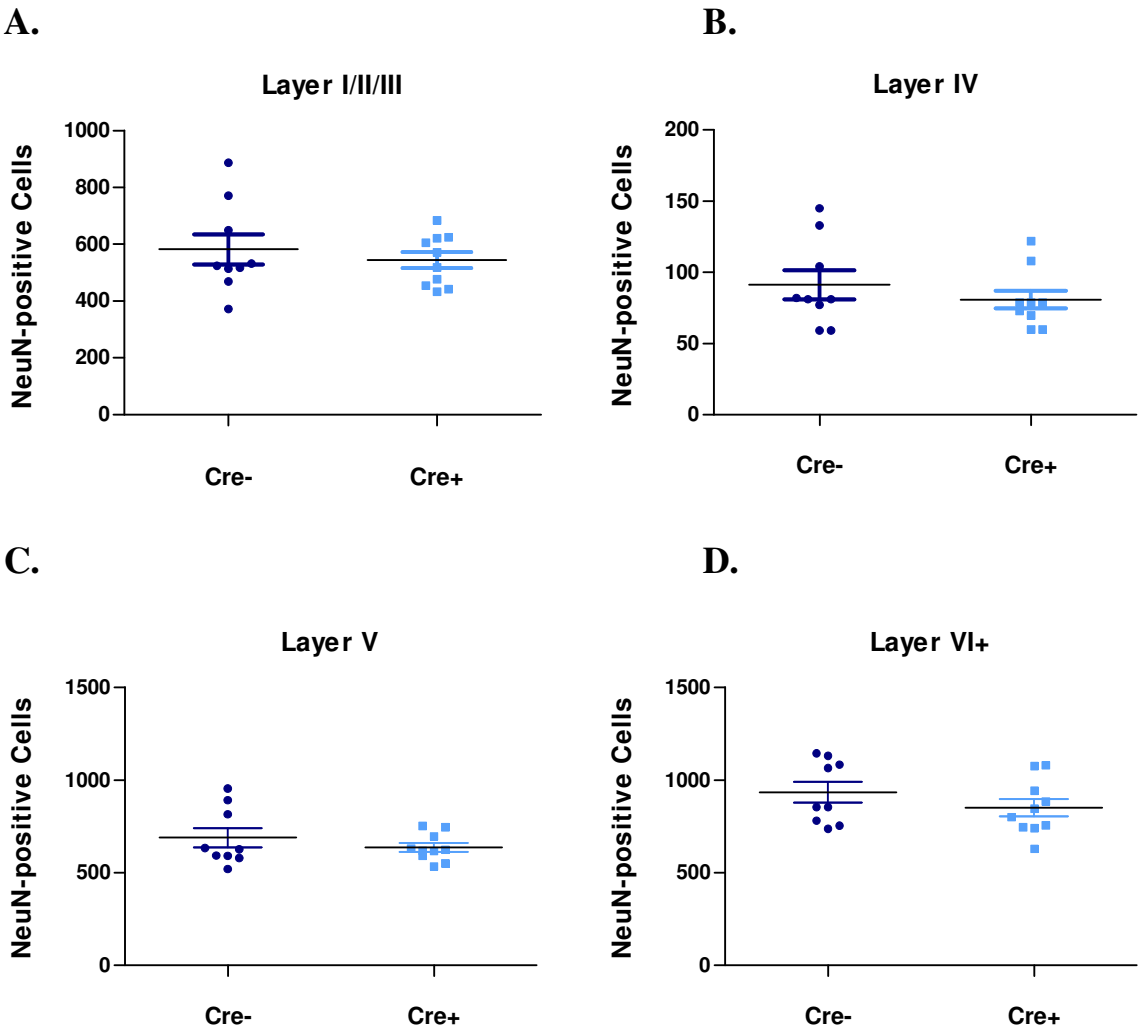


Figure 6. Number of NeuN-positive cells in individual layers of the cortex

Cre- represents TAK1$^{fl/fl}$ control mice and Cre+ represents TAK1^{nko} mice. Sections corresponding to section number 47 in the Paxinos Brain Atlas were stained for NeuN by DAB immunohistochemistry and quantified using ImageJ software. Statistic was performed using Students t-test A. p= 0.5282, B. p =0.3822 C. p=0.3591 D. p=2718. No significant difference was observed between the layers of Cre- (n=10) and Cre+ (n=9) male and female mice.

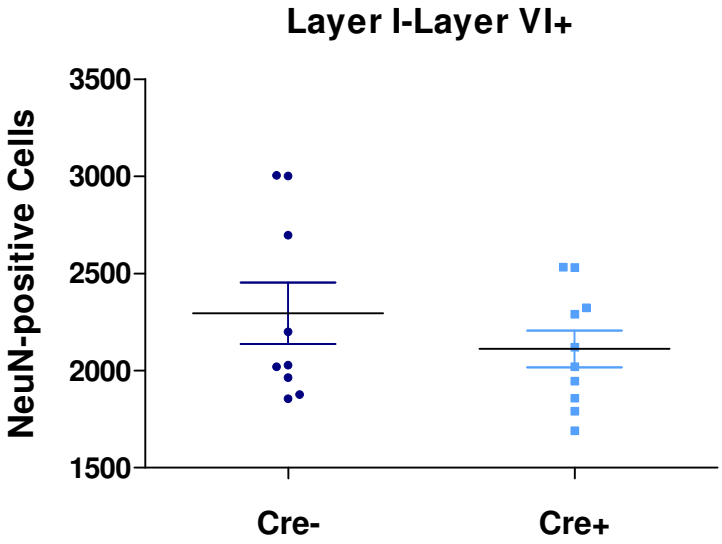


Figure 7. Number of NeuN-positive cells in all layers of cortex

Cre- represents TAK1$^{fl/fl}$ control mice and Cre+ represents TAK1^{nko} mice. The cryosections of brains were stained for NeuN by DAB immunohistochemistry and counted using ImageJ software. Statistical analysis was performed using Students t-test. p=0.3246. No significant difference was observed in both Cre- (n=10) and Cre+ (n=9) male and female mice in all layers together.

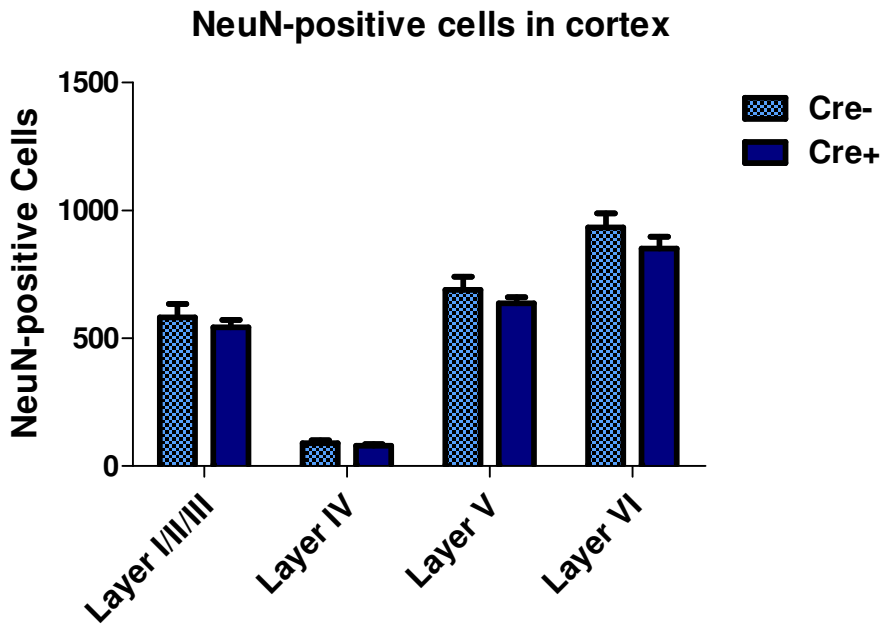


Figure 8. Number of NeuN-positive cells in individual layers of the cortex

Cre- represents $TAK1^{fl/fl}$ control mice and Cre+ represents $TAK1^{nko}$ mice. The numbers of NeuN-positive cells were counted in the regions of cortex: Layer I/II/III, Layer IV, Layer V and Layer VI. These regions were defined by comparison with section number 47 of the Paxinos Brain Atlas (note: region Layer IV is much smaller region than the other regions). Statistical analysis was performed using repeated measures-2-way ANOVA; Genotype: $F(1,51)=1.03$, $p=0.3246$; Layer: $F(3,51)= 365.43$, $p<0.0001$, no interaction. In each region of cortex measured for neuronal cells, no significant differences were observed in both the Cre+ (n=10) and the Cre- (n=9) male and female mice.

4.3. Hippocampus

In order to analyze the number of neurons in the hippocampus, we selected section 68 of the Paxinos Brain Atlas and compared the number of neurons observed in the frame in the brains of $TAK1^{nko}$ and $TAK1^{fl/fl}$ mice.

In accordance with brain-atlas, we divided the hippocampal area into three regions- CA1, CA2, CA3 (Fig. 9, 10).

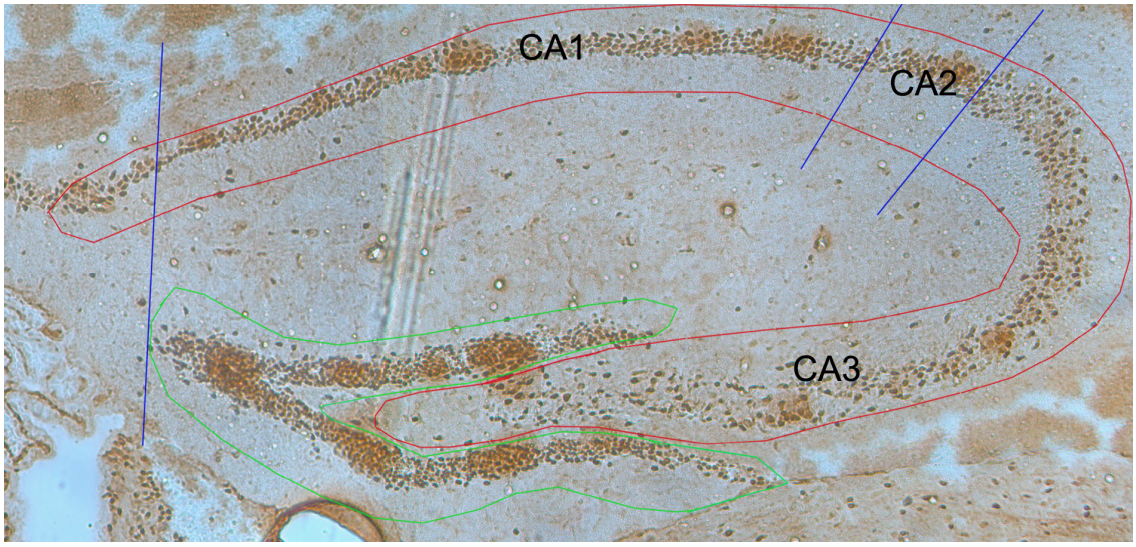
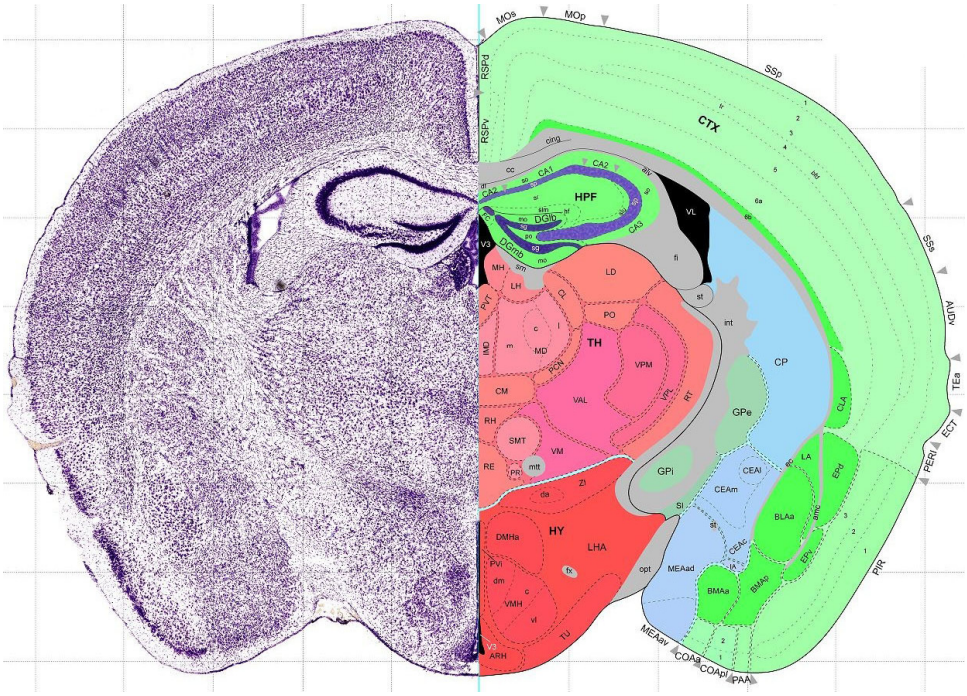


Figure 9. Example of stained section with antibody anti-NeuN (1:1000) by 10x magnification

DAB immunohistochemistry was used to visualize the neurons in hippocampus. The neurons were stained by using the anti-NeuN antibody (1:1000). Region of hippocampus was divided into CA1, CA2 and CA3 regions in accordance with Paxinos Brain Atlas. The neurons were counted in area in red contour limited by blue line.

A.



B.

Figure 10. Example of brain section for counting of neurons of the hippocampus

(A) Reference section from brain atlas. (B) Stained section with anti-NeuN antibody (1:1000) and DAB solution. The region of hippocampus is overlapped with a countour of a model section number 68 according to Paxinos Brain Atlas.

Quantification of neurons in section number 68 (number according to Paxinos Brain Atlas) revealed no difference in the numbers of neurons of $TAK1^{nko}$ and control mice (Fig. 11). All layers of the cortical sections were analyzed individually and no significant difference in neuronal numbers was observed. In CA1 region Cre+ mice seem to have lower number of neurons, whereas in CA2 and CA3 region Cre- mice seem to have lower number of neurons. However, difference in number of neurons between both of groups is minimal.

In addition, we compared the total number of neurons in a determined area of the hippocampus and observed huge variation in the number of neurons in between the groups. We found, that no significant difference is observed between both groups (Fig. 12).

To evaluate whether the number of neurons in the individual regions between both, Cre- and Cre+ mice are different, we tested the groups in 2-way ANOVA (Figure 13). No significant difference was found between the individual layers and groups.

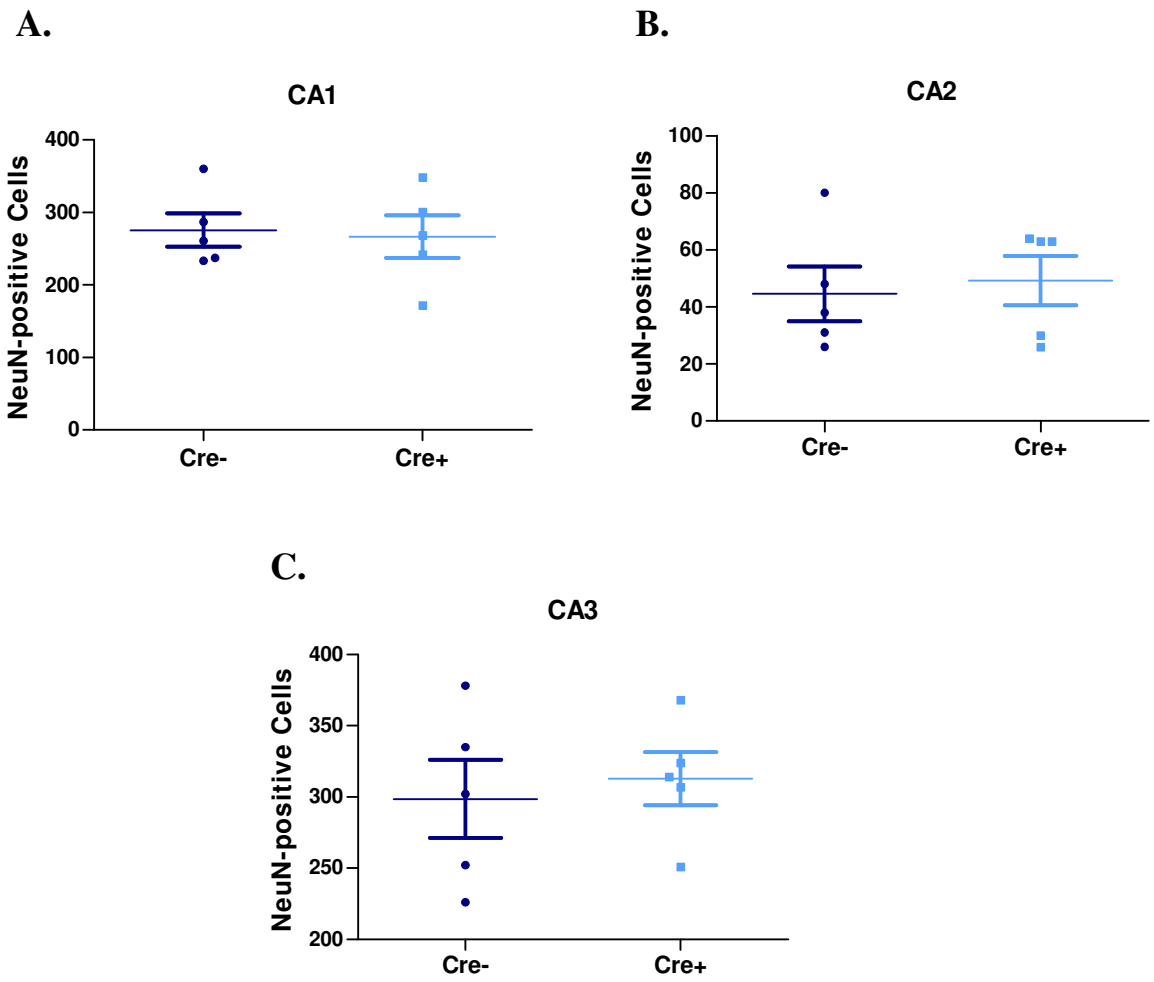


Figure 11. Number of NeuN-positive cells in individual regions of the hippocampus

Cre- represents $TAK1^{fl/fl}$ control mice and Cre+ represents $TAK1^{nko}$. Neurons of hippocampal sections were stained for NeuN by DAB immunohistochemistry and quantified using ImageJ software. Statistic was performed using Students t-test.

A. p=0.1869 B. p=0.7313 C. p=0.6806. No significant difference in single regions was observed in both Cre- (n=5) and Cre+ (n=5) female mice.

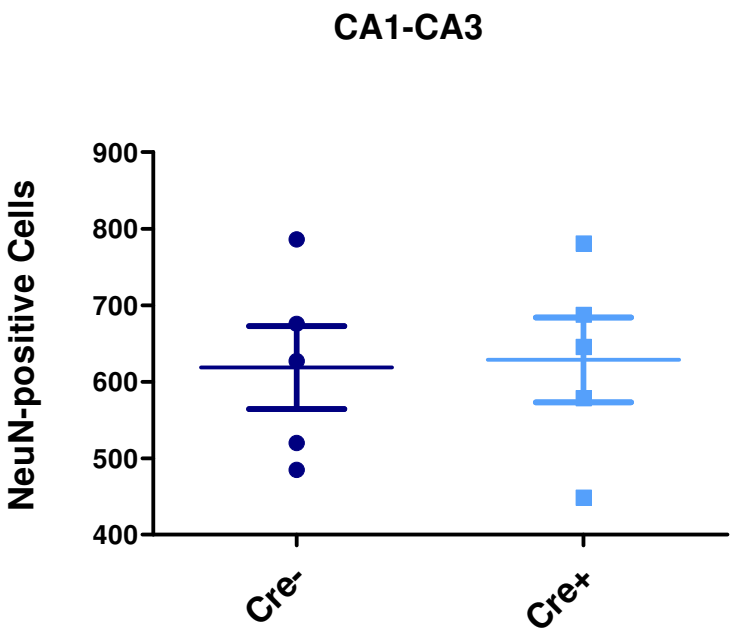


Figure 12 . Number of NeuN-positive cells in all regions of hippocampus

Cre- represents TAK1$^{fl/fl}$control mice and Cre+ represents TAK1^{nko}. The cryosections of brains were stained for NeuN by DAB immunohistochemistry and counted using ImageJ software. Statistical analysis was performed using Students t- test. p=0.9027. No significant difference was observed in both Cre- (n=5) and Cre+ (n=5) female mice in all regions together.

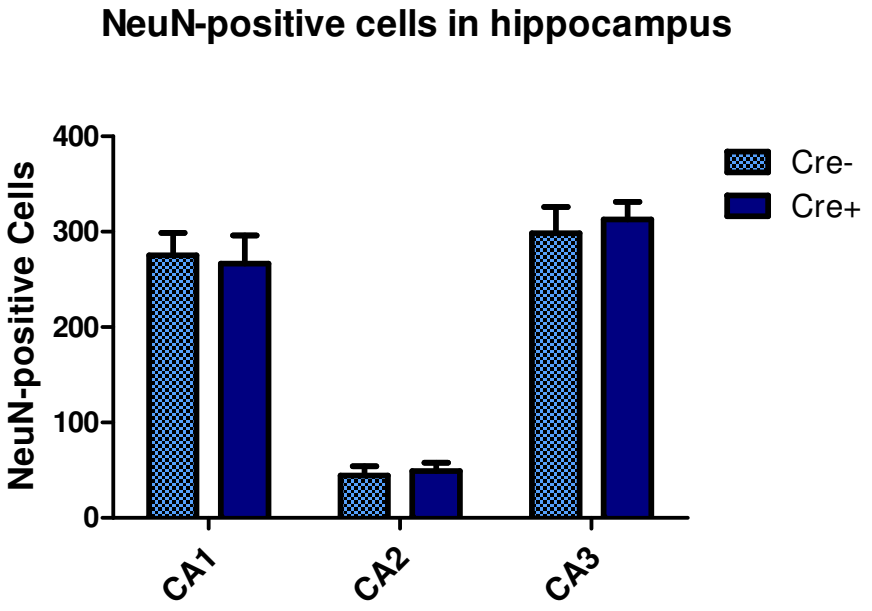


Figure 23. Nummer of NeuN-positive cells in each anatomical region of the hippocampus.

Cre- represents $TAK1^{fl/fl}$ control mice and Cre+ represents $TAK1^{nko}$ mice. The numbers of NeuN-positive cells were counted in the CA1, CA2 and CA3 regions. These regions were defined by comparison with section number 47 of the Paxinos Brain Atlas (note: region CA2 is a much smaller region than CA1 and CA3). Statistical analysis was performed using repeated measures-2-way ANOVA; Genotype: $F(1,24)=0.04$, $p=0.8517$; Layer: $F(2, 24)=88.12$, $p<0.0001$, no interaction. In each region of hippocampus measured for neuronal cells, no significant differences were observed in both the Cre- (n=5) and the Cre+ (n=5) female mice.

5. DISCUSSION

It has previously been reported that TAK1 is critical for early developement of neuronal tube (Shim et al. 2005). Here, we generated transgenic mice that were deficient for TAK1 in neurons after birth. Data from our group show that TAK1 plays a role on neuronal survival *in vitro*. The neurons deficient of TAK1 showed higher amount of apoptosis than wild-type neurons. Moreover, *in vivo* studies also showed that TAK1 is essential for the survival of different cell types. For instance, epidermal-specific deletion of TAK1 leads to massive apoptotic cell death (Omori et al. 2006). TAK1 has also an important function for the survival of both hematopoietic cells and hepatocytes due to apoptotic cell death observed in TAK1 knockout mice (Tang et al. 2008). Recent studies claimed that TAK1 is important for the survival of hepatocytes. Conditional ablation of TAK1 in liver parenchymal cells sensitizes hepatocytes to apoptosis as well as necrosis (Bettermann et al. 2010).

In contrast, we did not find any significant difference in the number of neurons between both $TAK1^{nko}$ and TAK1 floxed control mice. It is possible that TAK1 might mediate a survival signal for many tissue cells, although different cell types may exhibit different sensibility to the absence of TAK1.

Notably, mice lacking TAK1 in liver parenchymal cells showed stronger phosphorylation of the other MAP3 kinase TAO2. These mice also exhibit hyperactivation of JNK (Bettermann et al. 2010). In line with this study, we cannot exclude, that absence of TAK1 in neurons might be compensated by increased activation of the other MAP3 kinases. Otherwise, potential increased level of MAP3 kinases might contribute to increased JNK phosphorylation and pro-survival effect of JNK in neurons might be more significant than its pro-apoptotic effect.

NF-κB is a key regulator of programmed cell death. NF-κB is well known for its anti-apoptotic function (Barkett and Gilmore 1999; Dutta et al. 2006) as well as for its pro-apoptotic function (Inta et al. 2006). Thus, survival or fate of cells depends on a defined ratio of regulated target genes. Deletion of TAK1 leads to decreased activation of NF-κB (Shim et al. 2005) but NF-κB could be activated by other pathways (Senftleben et al. 2001). We found, that absence of TAK1 in neurons does not have any significant effect on their survival in comparison to control mice. Thus, pro-survival genes in neurons may be induced by other pathways of NF-κB, independent on TAK1.

It has been shown, that NF-κB plays a role in cerebral ischemia (Ridder and Schwaninger 2009), which is one of leading cause of mortality. Therefore, it is important to understand the molecular mechanisms of cerebral ischemia. It is also important to elucidate the physiological role of each member of the pathways that are involved in cerebral ischemia. In this study, we focused on the role of TAK1 in neuronal survival. We did not find any significant difference in number of neurons between mice that was deficient for TAK1 in neurons and the control group. However, we cannot exclude the possibility, that TAK1 plays an important role in neuronal survival. Further study may reveal the role of TAK1 in neurons to give us new insights into the complex molecular mechanisms of different pathways in the brain.

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Figure 2. *The Jackson Laboratory* [online]. Illustration from: http://cre.jax.org/introduction.html.